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Tissue engineering of small caliber vascular grafts ☆

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Abstract

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Keywords: Tissue engineering; Vascular grafts; Pulsatile flow

1. Introduction

Atherosclerotic vascular disease such as coronary artery disease and peripheral vascular disease, still is the largest cause of mortality in the western societies [1]. Surgical treatment of atherosclerosis began in 1952, when Voorhees et al. postulated to replace diseased blood vessels by synthetic fabric [2]. This led to the widespread clinical use of Dacron (polyethylene terephthalate (PET)) and Teflon (expanded polytetra-fluoroethylene (ePTFE)) grafts in cardiovascular surgery [3,4]. However, small-diameter grafts (i.e. I.D. ≤6 mm) of both Dacron and Teflon failed rapidly due to occlusion [5,6] and when used to bypass arteries showed rates of thrombosis greater than 40% after 6 months [7]. Recent strategies to increase graft patency included protein coatings to minimize blood/biomaterial interactions [8,9] and luminal seeding of the synthetic grafts with various cell types to create a living hemocompatible lining [10,11]. Although these reports have shown promising initial results, so far they did not come up with an 'ideal' solution. Synthetic grafts may still induce low-level foreign body reaction and chronic inflammation [12], and as artificial materials they are at an increased risk for microbial infections [13].

In an attempt to overcome these limitations cardiovascular tissue engineering is a new multidisciplinary approach to create completely autologous, living replacement structures such as heart valves and blood vessels. As viable structures, tissue-engineered blood vessels represent a responsive and self-renewing tissue with the inherent potential of healing and remodelling according to the needs of the specific environment. Generally, most tissue engineering approaches rely on bioabsorbable synthetic or natural materials as a scaffolding to provide a temporary biomechanical profile until the cells produce their own extracellular matrix. This has been

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especially true for tissue-engineered vascular grafts where 'hemodynamic' competence and suturing characteristics are critical. However, previous tissue engineering concepts to create vascular grafts have been limited by a lengthy in vitro tissue formation and structural and mechanical immaturity of the constructs at the time of in vivo implantation. We hypothesized, that providing a 'biomimetic' in vitro environment will accelerate tissue formation and yield more mature, implantable vascular grafts. Therefore, we developed a novel pulse duplicator system in which the vascular constructs were grown under controlled pulsatile flow and pressure conditions.

2. Materials and methods

2.1. Bioabsorbable vascular scaffolds

Non-woven polyglycolic-acid mesh (PGA, thickness: 1.0 mm, specific gravity: 69 mg cm^{-3}, Albany Int.) was coated with a thin layer of poly-4-hydroxybutyrate (P4HB, MW: 1×10^6, PHA 4400, TEPHA Inc., Cambridge, MA) by dipping into a tetrahydrofuran solution (1% wt/vol. P4HB). Following solvent evaporation, a continuous coating and physical bonding of adjacent fibers was achieved. P4HB is a biologically derived rapidly absorbable biopolymer which is not only strong and pliable, but also thermoplastic (Tm 61°C) so it can be moulded into almost any shape. From the PGA/P4HB composite scaffold material tubular scaffolds with an inner diameter (I.D.) of 0.5 cm and a length of 4 cm were fabricated using a heat application welding technique. The constructs were then cold gas sterilized with ethylene oxide (Fig. 1).

2.2. Cell isolation and culture

Endothelial cells were obtained from segments of ovine carotid artery (2–3 cm) using a collagenase instillation technique. The vascular tissue was incubated for 20 min at 37°C, 5%CO_2 in DMEM containing 0.2% collagenase type A (Boehringer Mannheim) and 1% bovine serum albumin (HyClone) and thereafter cultured in tissue culture flasks (Corning Inc.) using Medium 199 (Gibco) supplemented with 10% fetal bovine serum (HyClone), penicillin, streptomycin (Gibco) and 50 IU/ml heparin (Promega). To obtain myofibroblasts, the remaining de-endothelialized vessel segments were minced and cultured on P100 dishes (Corning) in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (HyClone), penicillin, streptomycin (Gibco). After migration of the myofibroblasts onto the dishes (after 5–7 days), the cells were serially passaged and expanded in a humidified incubator at 37°C and 5% CO_2. Sufficient cell numbers for cell seeding were obtained in pure culture after 21–28 days.

2.3. Cell seeding and in vitro culture in a pulse duplicator system

Myofibroblasts ($4.5–5.5 \times 10^6$ per cm^2) were seeded onto the inner surfaces of the vascular scaffolds and cultured in static nutrient medium (DMEM, Gibco) for 4 days in a humidified incubator (37°C, 5% CO_2). Thereafter the constructs ($n = 24$) were seeded with endothelial cells ($1.5–2.0 \times 10^6$ per cm^2 inner vascular surface), transferred into a pulse duplicator system ('bioreactor', Fig. 2) and grown under gradually increasing nutrient media flow and pressure conditions (125 ml/min at 30 mmHg to 750 ml/min at 55 mmHg) for 4 ($n = 4$), 7 ($n = 5$), 14 ($n = 5$), 21 ($n = 5$), and 28 ($n = 5$) days. Controls ($n = 24$) were grown in static nutrient media accordingly. The media was changed every 7 days.

2.4. Microstructure

A representative portion of each vascular construct was examined histologically by hematoxylin & eosin (H&E) and factor VIII stain. Additional samples were fixed in glutaraldehyde 2% (Sigma) for scanning electron microscopy (SEM).

2.5. Tissue analysis

Biochemical assays were performed for analysis of cellular and extracellular components of the neo-tissue. Total DNA was isolated and purified by sequential organic extractions with phenol and phenol/chloroform/isoamyl alcohol and quantified by spectrophotometry [14]. For determination of total collagen content, tissue was completely acid-digested and total 5-hydroxy-proline was measured [15].

The suture retention strength of the tissue-engineered vascular grafts was measured using a mechanical tester (Instron, Instron Corp., Canton, MA) and 4–0 prolene suture material. The testing was carried out at room temperature with a 100 Newton (N) load cell. The cross head speed was 20 mm/min. Rectangular shaped specimens of 20 mm gauge length and a single suture (set at 5 mm distance from the specimens edge) were measured and tensile force was applied until complete rupture. Moreover, the burst strength was measured by cannulation of the vascular constructs on a specially designed system. They were pressurized with phosphate buffered saline (PBS Dulbecco's, Gibco) and the hydrostatic pressure was increased by 5 mmHg steps until vessel failure.

2.6. Statistics

Result data were expressed as mean ± standard error of the mean. We used SPSS 8.0 software for statistical analysis. An unpaired *t*-test (Student's *t*-test) was performed, considering a *P*-value <0.05 as statistically significant.

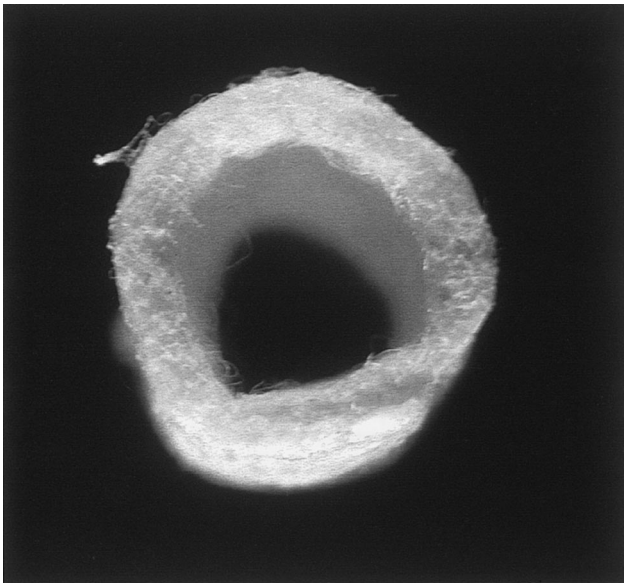

Fig. 1. Vascular scaffold for tissue engineering fabricated from bioabsorbable PGA/P4HB polymer mesh using a heat application welding technique. Note the highly porous luminal surface of the vascular graft (inner diameter (I.D.) = 0.5 cm).

3. Results

3.1. Tissue microstructure

Histology of the tissue-engineered vascular constructs revealed viable cellular tissue in all samples. There was early organization of the tissue in a layered fashion with a dense inner (luminal) layer and lesser cellularity in the outer portions after 21 days in the pulsed vascular grafts, whereas the static controls showed less tissue formation and organization at all time points. Factor VIII positive cells were detectable on the luminal surfaces but a confluent layer of endothelial cells was not seen. SEM demonstrated dense tissue and a confluent smooth surface with cell orientation in the direction of the flow after 14 days whereas the controls showed a less homogeneous surface at all time points (Fig. 3). Biodegradation of the polymer scaffold was detected by hydrolytic breakage and fragmentation of the polymer fibers.

3.2. Tissue analysis

The DNA- and Hydroxyproline assays showed a continuous increase of cell mass and collagen content to 21 days compared to significantly lower values in the static controls (DNA, $P = 0.004$; Hydroxyproline $P < 0.001$) (Fig. 4).

3.3. Mechanical properties

There was a continuous increase of burst strength of the vascular grafts grown in pulsatile flow conditions up to more than 300 mmHg in contrast to a substantial decrease over time in the static controls reaching a significant difference among the groups after 21 days ($P < 0.005$). Similarly the suture retention strength of the pulsed vessels was more than 5 times higher than of the controls. Both groups showed a decrease in wall thickness over time (Table 1).

4. Discussion

The clinical use of artificial vascular grafts (Dacron, ePTFE) to treat atherosclerotic disease represents a tremendous progress in modern cardiovascular surgery and has saved and improved millions of lives. Larger diameter vascular grafts have shown good long-term function for more than 10 years post-implantation [4]. In contrast, small caliber artificial grafts (i.e. I.D. <6 mm) still tend to fail rapidly due to thrombotic occlusion [5,6]. To address this problem, several approaches to modify vascular graft surfaces such as seeding of endothelial cells, impregnation or coating with biologically relevant proteins (e.g. collagen, albumin, fibronection, and laminin), and additives such as anticoagulants, growth factors and antibiotics have been tested. Although significant advances with improved patency rates have been made, prin-

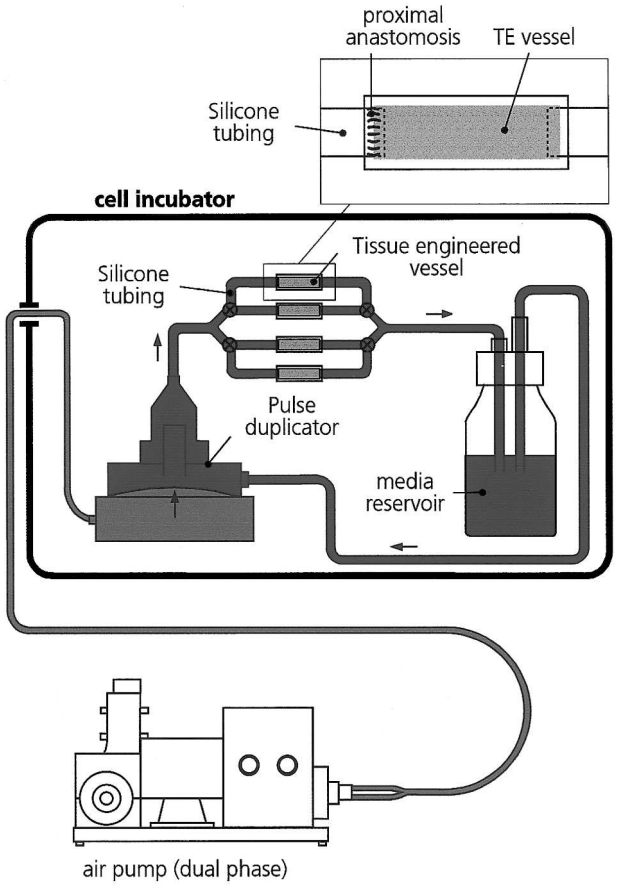

Fig. 2. Schematic description of the pulsatile in vitro flow system (bioreactor). The pulsatile flow of the nutrient media is generated by periodical expansion of a highly elastic membrane, de- and inflated by an air pump. In the setting of the current study four tissue-engineered vascular grafts were grown simultaneously.

cipally all these approaches comprise the implantation of artificial, non-living materials into the body associated with a substantial risk of foreign body reactions [13] and microbial infections [14].

Tissue engineering applies the principles and methods of engineering to biological sciences in an attempt to create viable replacements of deficient natural structures [16]. The option of creating living blood vessels from autologous cells offers many potential advantages compared to traditional synthetic implants such as the absence of thrombotic occlusion, the ability to grow, and the inherent potential of healing and remodelling according to the needs of the specific environment.

In 1986, Weinberg and Bell were the first to produce a completely biological tissue-engineered vascular grafts from animal collagen gels and bovine vascular cells [17].

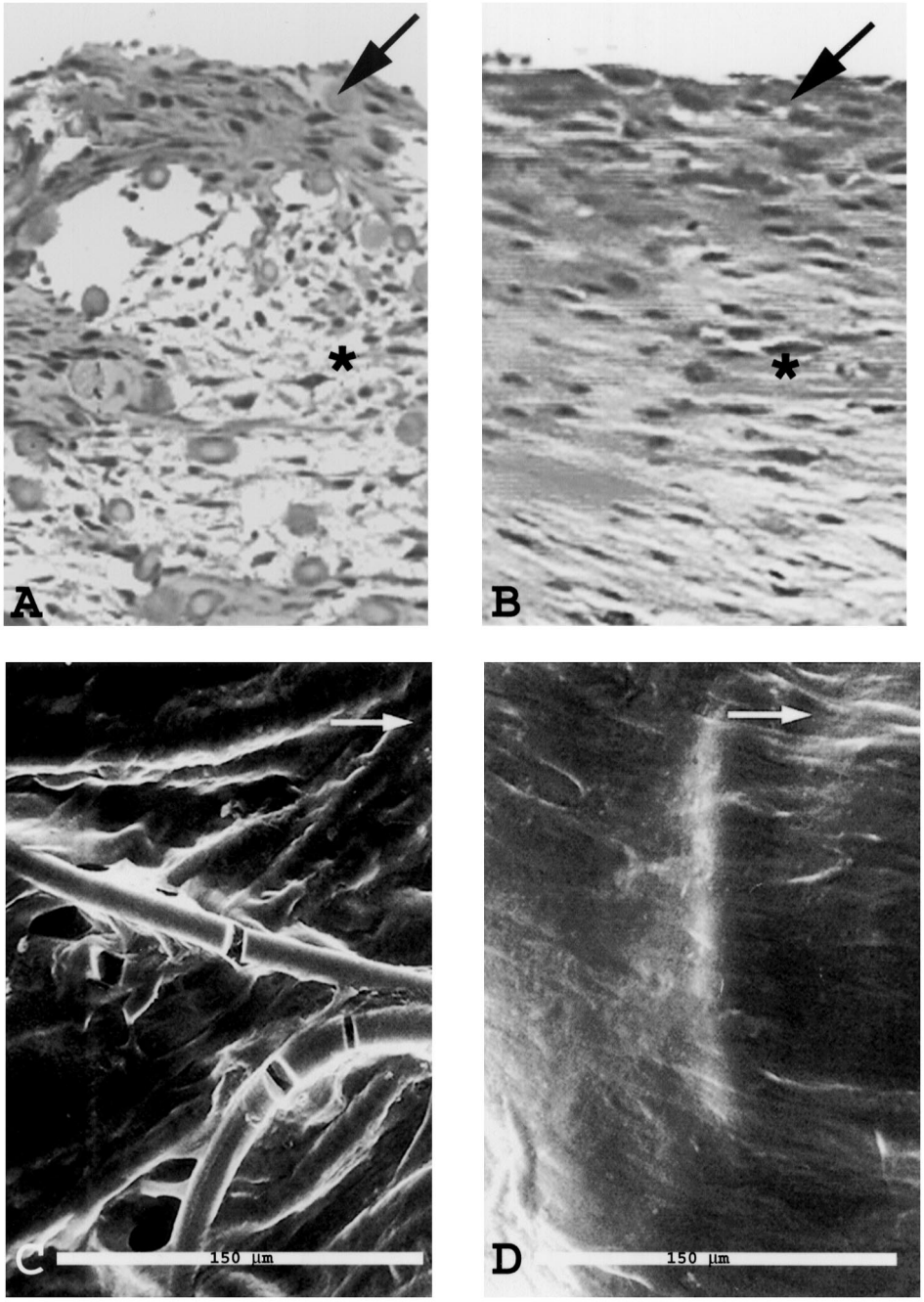

Fig. 3. Upper panel: Histology (H&E, ×100) of tissue-engineered vessels at 21 days in vitro culture. Note the more organized tissue structure of the pulsed vascular graft (B) with a dense luminal layer (↓) and an outer portion with tight extracellular matrix formation (*) compared to the static control (A). Lower panel: Scanning electron microscopy revealed a smooth, confluent luminal surface of the pulsed graft (D) in contrast to a less homogenous surface in the static control (C). Note the orientation of the cells in the direction of flow in D (white arrow = direction of in vitro flow).

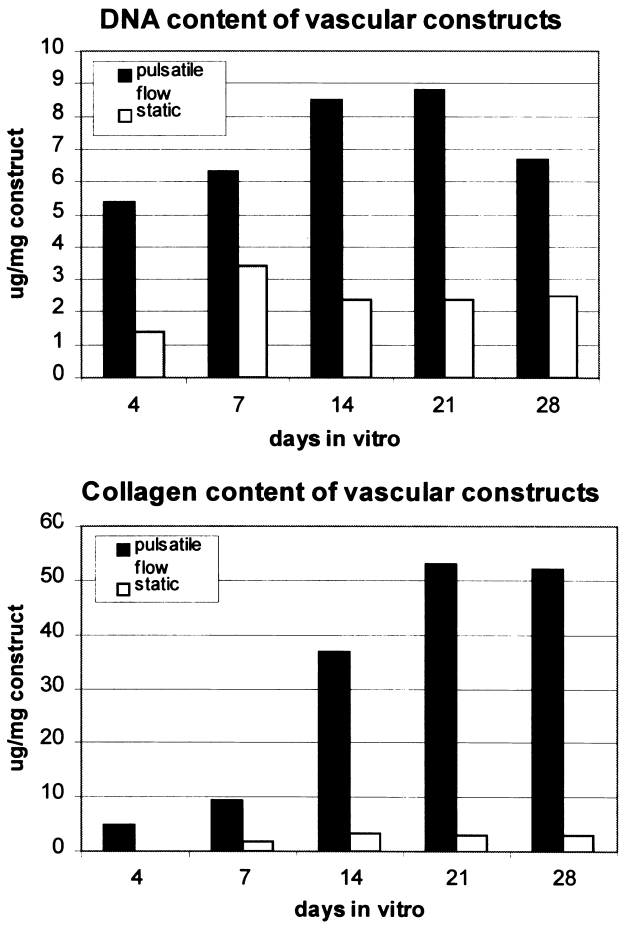

Fig. 4. Cell- and collagen content of the tissue-engineered vascular grafts (pulsatile flow vs. static in vitro culture conditions).

Unfortunately, the mechanical properties of these grafts were insufficient for in vivo implantation and even reinforced with a Dacron mesh failed to show adequate burst strength. Other groups using a similar approach with human collagen and human vascular cells encountered the same mechanical limitations [18]. Recently, L'Heureux et al. reported the feasibility of tissue-engineered blood vessels with good mechanical properties based exclusively on the use of cultured human cells without any synthetic or exogenous biomaterials [19]. A disadvantage of this approach was the fact that it took minimum 3 months until implantable grafts were produced. Another excellent concept reported by Niklason et al. used a biodegradable PGA mesh as scaffolding for vascular grafts which were wrapped around a silicone tubing, seeded with bovine vascular cells and cultured for 2 months [20]. In vitro pulsatile stress was indirectly applied to the construct by perfusing the silicone tubing resulting in a periodical radial distention of the vascular grafts.

In the present study we introduce a novel pulsatile in vitro system to grow seeded vascular constructs under 'biomimetic' flow conditions. In contrast to previous studies the pulsed flow of nutrient media was directed immediately through the vascular lumen, thereby generating direct shear stress to the luminal surface as well as periodical radial distension of the vessel wall. We anticipated, that exposure of the developing vascular tissue to physical signals similar to those encountered in vivo might result in accelerated tissue maturation and formation of mechanically stable, implantable vascular grafts.

We found that in comparison to standard static culture conditions there was advanced tissue formation in an organized, layered fashion. In contrast to the controls, the tissue was of compact composition without signs of a loose central area. It is known from previous tissue engineering approaches utilizing PGA scaffolds that the inner areas of the generated neo-tissues frequently showed signs of cell necrosis due to sub-optimal nutrient supply as well as an hydrolytic degradation-related acidic local milieu. The absence of this phenomenon in the current study may be explained by an improved nutrient media tissue supply and in parallel an increased 'wash out' of the local acidotic tissue milieu through utilization of our newly developed pulsatile flow system. Regarding the luminal morphology of the tissue engineered vascular grafts, there was a smoother, more confluent surface in the pulsed grafts with homogenous cell orientation in the direction of the flow. However, although factor VIII positive endothelial cells were detected on the luminal surfaces, they were not completely confluent. This may result from the fact that the vascular grafts were exposed to increased in vitro flow at a too early stage of the tissue maturation process, resulting in a partial detachment and 'wash-out' of the endothelial cells.

The biochemical matrix analysis showed a continuous increase of cell mass and collagen content in the pulsed vascular grafts resulting in significantly higher values compared to the non-flow controls after 7 days. Collagen

Table 1
Mechanical properties of the tissue-engineered vascular grafts (pulsatile flow vs. static in vitro culture conditions)

Time in vitro (days)	Burst strength (mmHg)		Suture retention (g)		Wall thickness (mm)	
	Pulsatile flow	Control (static)	Pulsatile flow	Control (static)	Pulsatile flow	Control (static)
7	177.5 (±10)	178.8 (±4)	74.5 (±4)	67.3 (±5)	0.85 (±0.08)	0.98 (±0.08)
14	240.0 (±28)	110.0 (±18)	56.8 (±5)	42.0 (±5)	0.75 (±0.05)	0.63 (±0.13)
21	262.5 (±26)	90.0 (±8)	64.8 (±3)	25.0 (±8)	0.63 (±0.04)	0.50 (±0.05)
28	326.3 (±24)	50.0 (±5)	64.3 (±5)	12.0 (±3)	0.73 (±0.04)	0.50 (±0.1)

represents the key extracellular matrix component for mechanical stability and therefore is critical for the surgical implantability of the tissue-engineered constructs. In accordance with the superior extracellular matrix formation, the mechanical characteristics of the pulsed vascular grafts were more favourable regarding burst strength and suture retention strength, resulting in properties appropriate for surgical implantation after 3 weeks. In contrast, the static controls showed a continuous loss of mechanical properties in parallel to the biodegradation-related decrease of the mechanical strength of the scaffold material. Since the scaffold was supposed to provide only a temporary biomechanical profile until the cells produce their own matrix proteins the structural integrity and biomechanical profile of the tissue-engineered vessels ultimately depended on this matrix formation. The observed decrease of vascular wall thickness in both groups is a known phenomenon in tissue engineering reflecting a certain shrinkage of the constructs during biodegradation of the polymer scaffold material.

These preliminary results demonstrate that acceleration of the in vitro tissue formation and maturation of tissue-engineered small caliber vascular grafts is feasible using a 'biomimetic' pulsatile flow system. The grafts showed a vessel-analogous tissue organization and mechanical properties appropriate for surgical implantation. Additional studies focusing on in vivo application will have to be performed to further validate the presented concept. Finally, optimization of the in vitro conditions with regard to growth factors, growth inhibitors and pressure loading conditions are areas for future studies.

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